

DEVELOPMENT OF A THREE-DIMENSIONAL *IN VITRO*
MODEL TO STUDY THE EFFECT OF VITAMIN D ON BONE
METASTATIC BREAST CANCER

By

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ABSTRACT

Breast cancer has a high prevalence among women and most patients suffer from metastasis to bone. The mechanisms involved in breast cancer bone metastasis are poorly understood. Three-dimensional (3D) tissue culture systems are becoming a focus of biomedical engineering. The 3D cultures can mimic the *in vivo* microenvironment and have much greater biological relevance than the traditional two-dimensional (2D) monolayer cell culture systems. There is a need for developing reliable *in vitro* models to study the mechanism of bone metastasis in breast cancer. We established a 3-dimensional (3D) collagen matrix tissue culture model to study metastatic breast cancer. Calcitriol or 1, 25 dihydroxyvitamin D₃ has anti-tumor effects by inhibiting cancer cell proliferation and inducing differentiation. There are few research studies investigating its role in metastasis. None of the research studies were based on bio-mimetic 3D models. We used this novel 3D tissue culture system to test 1, 25 dihydroxyvitamin D₃'s role in breast cancer bone metastasis. Our results demonstrate vitamin D pretreated breast cancer cells had significantly decreased ($p \leq 0.0001$) migration rate, in association with significantly decreased ($p \leq 0.05$) cell surface expression of CXCR4. This result proved the hypothesis vitamin D inhibits breast cancer bone metastasis by down regulating CXCR4 expression. Our data showed in the 3D system, breast cancer migration rate increased significantly compared with those in 2D system. In our pilot study, CXCL12 expression data suggest that the decrease in migration was not due to the CXCR4-CXCL12 axis because there was no significant difference ($p \geq 0.05$) of CXCL12 expression between 3D and 2D osteoblast cells. The CXCL12 studies needs to be validated with a

larger sample size. Alternatively, this observation suggests that there are other signaling pathways which influence the migration of breast cancer cells towards osteoblast cells and bone matrix.

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CHAPTER 1

INTRODUCTION

Background

Bone is one of the most preferential metastatic target sites for cancers including breast cancer. Most breast cancer patients will develop bone metastasis. It can cause severe pain, pathologic fractures, life threatening hypercalcemia, spinal cord compression, and other nerve compression syndromes [1]. Although the cancer incidence and mortality remain severe, the mechanisms of breast cancer bone metastasis are poorly understood. This makes it difficult to find effective chemotherapeutic agents. Based on some existing research studies, vitamin D could decrease the migration of breast cancer cells in two-dimensional (2D) models. To our best knowledge, no studies have used a three-dimensional (3D) model containing collagen matrix, which is an important component of bone, to study the inhibiting effect. To study the modulating effect of 1, 25 dihydroxyvitamin D₃, we compared the efficiency of the inhibiting effect of vitamin D between 2D and 3D models in this study.

Chemokines are believed to be associated with the metastasis of various types of cancers. The CXCL12/CXCR4 signaling axis is one of the well-known mediators of breast cancer metastasis. The CXCR4 expressing human breast cancer cells can easily home to the organs which has high expression of its ligand, CXCL12. The expression of CXCR4 in human specimens is also a marker for prognosis. In this study, we looked for the differences in the expression of CXCR4

between 1, 25 dihydroxyvitamin D₃ treated and untreated breast cancer cells. We also evaluated the expression of CXCR4 would change the expression of CXCL12 by osteoblast cells.

Statement of purpose

The purpose of this study was:

- (1) To establish a 3D *in vitro* tissue culture model;
- (2) To evaluate the efficiency of migration of breast cancer cells towards osteoblast cells;
- (3) To test the effects of 1, 25 dihydroxyvitamin D₃ in inhibiting osteotropic migration; and
- (4) To apply this model to study one potential mechanism of breast cancer bone metastases

Research questions

1. Does the matrix provided by the 3D microenvironment aid in forming a more efficient *in vitro* metastatic breast cancer model?
2. Can 1, 25 dihydroxyvitamin D₃ decrease the migration of breast cancer cells towards osteoblast cells cultured in collagen scaffolds in a 3D microenvironment?

CHAPTER 2

REVIEW OF LITERATURE

Introduction of breast cancer

Breast cancer has a high incidence every year, and is the primary cause of cancer death among women worldwide (1). Bone is one of the most preferential metastatic target sites for breast cancer. Up to 70% of breast cancer patients will develop bone metastasis (2). Bone metastasis can cause severe pain, pathologic fractures, life threatening hypercalcemia, spinal cord compression, and other nerve compression syndromes (3).

Bone metastasis of breast cancer is difficult to treat owing to its complexity. Current treatments, such as radiation therapy, bisphosphonates, chemotherapy and hormone therapy for bone metastatic breast cancer patient only improves the quality of life. If cancer is restricted or localized, radiation therapy is primary choice to shrink the tumor and provide pain relief. Systemic therapies such as chemotherapy or other drug therapies are usually given to advanced breast cancer patients because of the extent of spread of the tumor cells (2).

In advanced breast cancer, tumor cells must complete all the steps involved in the metastatic cascade: ① proliferation and invasion of cancer cells at primary site; ② intravasation, migration in the circulation and extravasation; ③ arrest in the bone marrow, egress from central sinus and attachment to bone surfaces; and ④ colonization of cancer cells and bone destruction(3,4). Bone metastases are often in two forms: osteolytic (bone-resorbing) and osteoblastic (bone-forming),

and most patients with breast cancer have osteolytic lesions (4).

Recent research focuses on breast cancer bone metastasis

The exact mechanism for preferential metastasis is still not well-established. Most studies are focused on elucidation of the mechanisms involved in breast cancer metastasis to bone. The bone microenvironment is endowed with unique biological features that enable circulating cancer cells to metastasize (6). Cancers that develop bone metastasis are able to migrate from their primary site, adhere to bone and thrive in unique bone environment and ultimately destroy bones. This is the basis of the “seed and soil” hypothesis that was put forward by Paget to explain the principle of metastasis (3). According to this hypothesis migrated cancer cells (seeds) metastasize only to those organs which provide a fertile microenvironment (soil) which is congenial for the growth of tumor cells (5). Many stem cells such as mesenchymal stem cell and monocytes which are hematopoietic, are important players in metastasis (4). The skeletal cells such as osteoblasts which have the function of bone formation, and osteoclasts which are mainly important for bone resorption, can produce and secrete growth factors, including transforming growth factor (TGF)- β , insulin-like growth factors I and II (IGF), fibroblast growth factors (FGFs), platelet-derived growth factors (PDGFs), and bone morphogenetic proteins (BMPs), as well as cytokines such as interleukin, macrophage colony-stimulating factor, and tumor necrosis factor- α (TNF- α). These growth factors and cytokines are involved in bone resorption via the receptor activation of nuclear factor- κ B ligand (RANKL) and its two known

receptors RANK and OPG (osteoprotegrin) (4-7). While RANK-RANKL axis is responsible for the maturation of osteoclasts which in turn facilitate bone resorption, OPG, the decoy receptor of RANKL, can effectively decrease osteolysis. Metastatic breast cancer cells secrete PTHrP (parathyroid hormone-related peptide) in the presence of some bone microenvironment growth factors such as TGF- β , and PTHrP promotes RANKL production and RANKL-RANK complex formation thus causing bone resorption. Breast cancer cells express a number of genes that may act as homing determinants to facilitate their migration to bone. These genes promote invasion and allow extravasation from capillaries within bone marrow, genes that confer responsiveness to chemotactic cues, and genes that allow adhesion to the bone extracellular matrix (2-4). For example, it was demonstrated that CXCR4, a chemokine receptor, is highly expressed in breast cancer cells relative to normal breast tissue. Stromal cell-derived factor 1 (SDF-1) or CXCL-12, the ligand for CXCR4, which is highly expressed in bone, determines the invasion and organ specific metastasis. CXCL12 can stimulate cancer cells via CXCR4 signaling, which induces chemotaxis and regulate their metastasis to bone (4, 8). Once breast cancer cells migrate to bone, they thrive in metabolically active bone environment and establish bone metastasis.

Most research studies on metastatic breast cancer are based on animal in vivo models, or two-dimensional (2D) in vitro models. However, these animal models are difficult to use for studying molecular mechanisms in the process of

invasion and migration of tumor cells. Sometimes the host animal tissues are not compatible with the implanted human tumors. The tumor response of animal models may not be the same as observed in humans (10-13). For 2D models, it is convenient to investigate cancer signaling pathways, but the cells grow in a different microenvironment from *in vivo* environments containing ECM (extracellular matrix) such as collagen and laminin. They attach to the bottom of the culture plates and turn out to be flat while in fact, cells are spherical *in vivo*, and dispersed between interstitial spaces of the ECM. The changes in morphology, as well as the differences in cell-cell and cell-matrix interaction from the cells in the physiological microenvironment, may decrease the significance of the results from 2D models. The development of hypoxia and signaling between cells residing within spatially distinct niches, also give rise to the ability of tumor aggression, yet these interactions can hardly be reflected in conventional 2D cell culture models (9).

Current three-dimensional (3D) cell culture models

During the recent years, research on cancer metastasis has gradually turned to 3D models because the 3D systems are believed to have greater biological relevance than their 2D counterparts. The purpose of this type of tissue culture system is to either implant cells in a 3D matrix scaffold; or use more than one type of cell or a tissue fragment in combination with another cell type in order to recreate the 3D cancer and bone microenvironment (9, 14). Pathi et al utilized bone mineral hydroxyapatite to set a 3D model with the neoplastic and bone

metastatic growth of breast cancer cells and reported that in the mineralized scaffold, breast cancer cells increased adhesion and penetration ability by the increased expression of fibronectin (15). Dhurjati et al. developed a 3D mineralized multi-cell-layer osteoblast in vitro system in a bioreactor and found breast cancer cells' adhesion, penetration, colony formation were similar to those seen in clinical samples. The breast cancer cells could also inhibit osteoblastic bone formation by down-regulating synthesis of collagen and osteocalcin. After colonization, osteoblast had increased expression of the pro-inflammatory cytokine IL-6 (16). Mastro et al. found that this 3D osteoblast system recapitulated the process of bone development in vivo including phenotypic change of osteoblasts into mature osteocytes, and increased expression of E11, DMP1, and sclerostin (17). Krishnan et al then compared such system with traditional 2D system and they did not see the tissue penetration or cells files (18). According to the above studies, the advantages of 3D models were due to the structure and orientation of extracellular matrix in 3D which cannot be present in 2D models. They played a critical role in supporting cell functions, transport and overall system feasibility.

Besides the mechanism studies, 3D models are currently used to evaluate therapeutic efficacy of anticancer agents in inhibiting tumor growth and metastasis. Paduch et al investigated the effects of vitamin D, tamoxifen and β -estradiol on breast cancer cell proliferation in 3D tumor spheroid culture system (19). Jin et al used a 3D model to investigate adenovirus-mediated antiangiogenesis and

oncolysis combination therapy on prostate cancer metastasis (15).

Currently there are very few studies that compare 3D models vs. 2D or in vivo animal models, or apply such models to evaluate the effect of potential anticancer agent on bone metastasis

Vitamin D's role in breast cancer bone metastasis

Vitamin D is well-known for its ability in inhibiting proliferation and promoting differentiation of cancer cells, yet there are few studies that report the effect of vitamin D on cancer metastasis. Some studies have demonstrated the potential of vitamin D in reducing prostate cancer metastasis by blocking gene Stat 3, which encodes interleukin-12, an osteoclastic metastasis induced factor (20, 21). Mork-Hansen et al demonstrated that 1, 25 dihydroxyvitamin D₃ inhibits invasion of breast cancer cells in 2D *in vitro* models, although the mechanism is not clear (22). Wang et al showed the potential of vitamin D in preventing cancer metastasis: low serum vitamin D level (< 20ng/ml) is prevalent in patients with breast cancer bone metastasis and is in conjunction with increased toxicity from bisphosphonate therapy (23). Vitamin D deficiency is associated with promoted growth of human breast cancer cells in the bones, with the secondary changes in the bone microenvironment and direct effects of it on tumor growth (10). Vitamin D analog EB 1089 decreased the total number of bone metastases, the mean surface area of osteolytic lesions, and tumor burden, and prolonged survival time (11).

Currently it is not clear whether vitamin D supplementation has a direct effect on metastasis since no significant changes in the bone resorption markers have

been observed although there was a significant reduction in the number of sites of pain (24).

There remain large gaps in our understanding regarding the biological targets or down stream signaling pathways modulated by vitamin D even in the models which proved vitamin D has positive effect in affecting metastasis. The inconsistent results, along with the elucidation of underlying mechanisms in the positive studies, make it necessary to clarify the role of vitamin D in breast cancer metastasis.

Future implications

In conclusion, an appropriate 3D model is a useful experimental tool. The advantages of it include: 1) cell morphology and signaling are more similar to those in physiological conditions than 2D cell culture models; 2) more applicable to implement rapid experimental manipulations than animal models. However, there are not many well-established 3D models, especially in breast cancer research. The existing ones vary in their ability to mimic in vivo tissue conditions according to their designs and the materials they use, and lack vasculature and normal transport of small molecules, host immune responses, and other cell-cell interactions. Therefore, the aim of the future study is to establish a new sustainable 3D tissue system to simulate the bone metastasis of breast cancer in vitro and then to utilize this system to screen different dietary agents for their anti-metastatic role.

CHAPTER 3

METHODS

The purpose of this study is to establish a new sustainable *in vitro* 3D tissue culture system to mimic the bone metastasis of breast cancer and use the system to test the effect of vitamin D on breast cancer bone metastasis.

Cell lines

Murine pre-osteoblast cell line, MC3T3-E1 was purchased from American Type Culture Collection (Manassas, VA), and human breast cancer cell line, MDA-MB-231, was generously provided by Roy Jensen (The University of Kansas Cancer Center, KU Medical Center, Kansas City)

Establishment of 2D and 3D *in vitro* models

Before cell culture, the collagen composite scaffolds (BD Biosciences, MA) were sterilized by immersing in 70% ethanol for 20 minutes followed by three washes with sterile HBSS (Hank's Buffered Salt Solution) (Fisher Scientific, IL) and primed by pre-incubating in complete medium (Dulbecco's Modified Eagle's Medium, with 10% fetal bovine serum) (Fisher Scientific, IL) overnight.

Twenty-four-well tissue culture plates were used to grow the cells while seeding density in each well was 5×10^5 MC3T3-E1 cells for 2D system, and the same amount per scaffold for 3D system. Each well contained one scaffold with cells. Cells were grown in complete medium, and were maintained in a 5% CO₂ incubator held at 37°C. The osteoblast cells were cultured for 15 days in complete medium supplemented with differentiation agents (10^{-7} M dexamethasone

(Sigma-Aldrich Co., MO), 50 μ M ascorbic acid (Sigma-Aldrich Co., MO), 10 nM 1,25 dihydroxyvitamin D₃ (Enzo Life Sciences, Inc.,PA), 10nM β -glycerol phosphate (Sigma-Aldrich Co., MO).

Osteoblasts conditioned media (OBCM) collection

On day 15, the osteoblasts growing with (3D) or without scaffolds (2D) were cultured in serum free complete medium. The OBCM from both 2D and 3D cultures were collected on day 16.

Breast cancer conditioned media (BCCM) collection

Sub-confluent (90%) MDA-MB-231 cells were pre-treated with 100nM 1, 25 dihydroxyvitamin D₃ for 48 hours. After 48 hours, serum free complete medium were used to culture the cells for another 24 hours. This serum free medium was collected as pre-treated BCCM. As control, serum free complete medium were used to culture the untreated sub-confluent breast cancer cells for 24 hours and were collect.

Pre-treated and untreated breast cancer cell collection

After collection of the conditioned media from pre-treated and untreated MDA-MB-231 cells, the cells were harvested and were used for cell migration assay.

Cell migration assay

Cell migration assay is based on the principle of Boyden chamber. The 24 well plates were used for migration assay. The wells with cells were designated as lower chambers, and PCF (polycarbonate) inserts with 8 μ m pore size (Millipore,

MA) as upper chambers. We seeded pre-treated and untreated MDA-MB-231 cells at a density of 1×10^5 cells per insert, respectively. The experimental combinations were as follows: untreated MDA-MB-231 in 2D system, untreated MDA-MB-231 in 3D system, treated MDA-MB-231 in 2D system, and treated MDA-MB-231 in 3D system. After 24 hours, the inserts were removed and stained with crystal violet.

Determination of cell migration

The cells on the inner surface of the membranes of each insert were swabbed. The membranes were immersed in 0.05% crystal violet solution for 30 minutes. The membranes were washed with distilled water three times and dried at ambient temperature. Pictures of migrated cells were taken with a digital microscope (Nikon, NY), and the number of migrated cells were counted and recorded.

Treatment of vitamin D pretreated breast cancer cells with OBCM

Pre-treated and untreated MDA-MB-231 cells were seeded in each well of 6 well plates at the density of 1×10^5 . The cells were cultured in 2D and 3D CM for 48 hours, respectively. The conditioned media consisted of one half volume OBCM and one half volume complete medium.

Detection of CXCR4 expression on the surface of breast cancer cells by Flow Cytometry

After culturing in OBCM for 48 hours, 1, 25 dihydroxyvitamin D₃ pre-treated and untreated MDA-MB231 cells were stained with PE-conjugated anti-CXCR4

mAb (FAB170P, Clone 12G5) (R&D Systems, Minneapolis, MN). PE-conjugated mouse IgG2a isotype-matched mAb (IC003P, R&D Systems) was used as the negative control. Flow cytometry was used to detect cell surface expression of CXCR4 and FlowJo (Tree Star, Inc., OR) was used to analyze the data.

Treatment of osteoblasts (2D vs. 3D) with BCCM

MC3T3-E1 cells were seeded at the density of 1×10^5 cells per well in 6 well plates and cultured in complete medium with differentiation agents for 1 day. To create the 3D system, each well had 2 scaffolds while the 2D system had MC3T3-E1 cells only. Complete media were replaced by pre-treated and untreated conditioned media to culture 2D and 3D osteoblasts for 24 hours, respectively. Conditioned media consisted of one half volume BCCM and one half volume complete medium.

Detection of CXCL12 expression on the surface of osteoblasts by quantitative real-time polymerase chain reaction (qRT-PCR)

Quantitative real-time PCR (qRT-PCR) was used to detect the expression of CXCL 12 by MC3T3-E1 in 2D and 3D system. RNA was obtained by the RNeasy minikit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Prior to isolating total RNA from cells embedded in 3D scaffolds, the scaffolds were chopped by microscissors and centrifuged at the speed of 10,000 x g for 5 minutes. The precipitation was used for RNA isolation. Quantitative real-time PCR was performed according to the protocol. The cycling condition was: 50.0 °C for 20 min, 95.0 °C for 12.5 min, 40 cycles of 94.0 °C for 15 sec, 57.0 °C for 20 sec,

72.0 °C for 1 min, 75 cycles of 55.0 °C for 10 sec, and 25.0 °C for hold. The transcript levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA.

Statistics

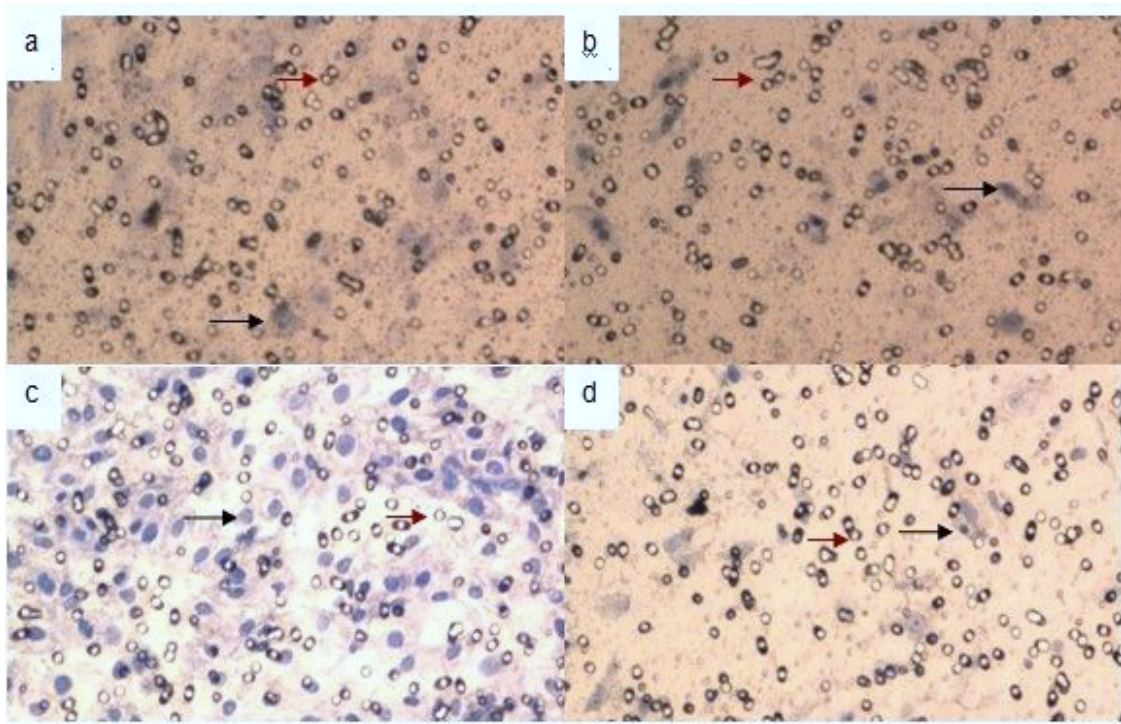
All data are presented as the mean \pm SD. Data were analyzed by two-sided Student's t test. Each experiment replicated three times and a minimum of three samples per experiment. A p value < 0.01 was considered statistically significant.

CHAPTER 4

RESULTS

Three-dimensional microenvironment increased the mobility of breast cancer cells, and 1, 25 dihydroxyvitamin D₃ decreased the mobility of breast cancer cells

To examine the effects of dihydroxyvitamin D₃ and 3D microenvironment on breast cancer metastatic ability, MDA-MB-231 were exposed to complete medium with or without 100nM vitamin D for 48 hours cultured in 2D system or 3D system. Breast cancer cell migration through a microporous membrane was assessed. We observed the exposure of both pre-treated and untreated breast cancer cells had significantly ($p \leq 0.0001$) increased migration ability in 3D system compared with those in 2D system. Approximately 100% more MDA-MB-231 cells per field of view were chemoattracted by the 3D tissue culture system. Vitamin D decreased breast cancer cell migration significantly in both 2D and 3D systems in vitro by about 50% (Figure 1, Figure 2).



a. 2D-1,25D3 b. 2D+1,25D3 c. 3D-1,25D3 d. 3D+1,25D3

Figure 1. Images of vitamin D pre-treated and untreated breast cancer migration in 2D and 3D microenvironment. 1, 25 dihydroxyvitamin D₃ pre-treated and untreated breast cancer MDA-MB-231 cells growing in inserts (upper chamber) were exposed to 2D or 3D systems (lower chambers) for 24 hours, and then migrated cells were stained with crystal violet. Black arrows indicate stained migrated MDA-MB-231 cells. Red arrows refer to the pores of the insert membranes. (a: untreated MDA-MB-231 cells in 2D osteoblast system; b: pre-treated MDA-MB-231 cells in 2D osteoblast system; c: untreated MDA-MB-231 cells in 3D osteoblast system; d: pre-treated MDA-MB-231 cells in 3D osteoblast system)

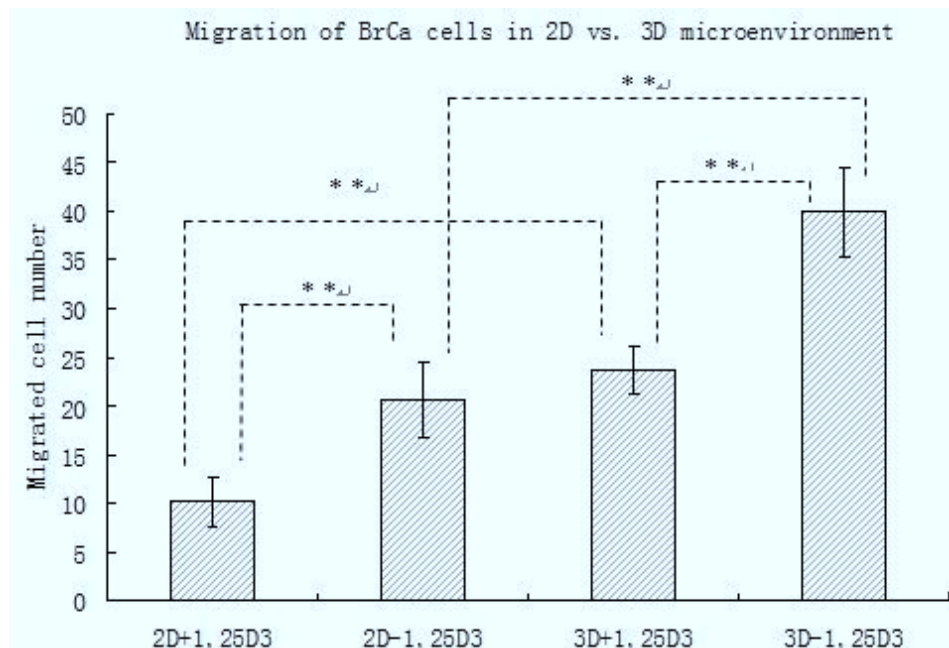


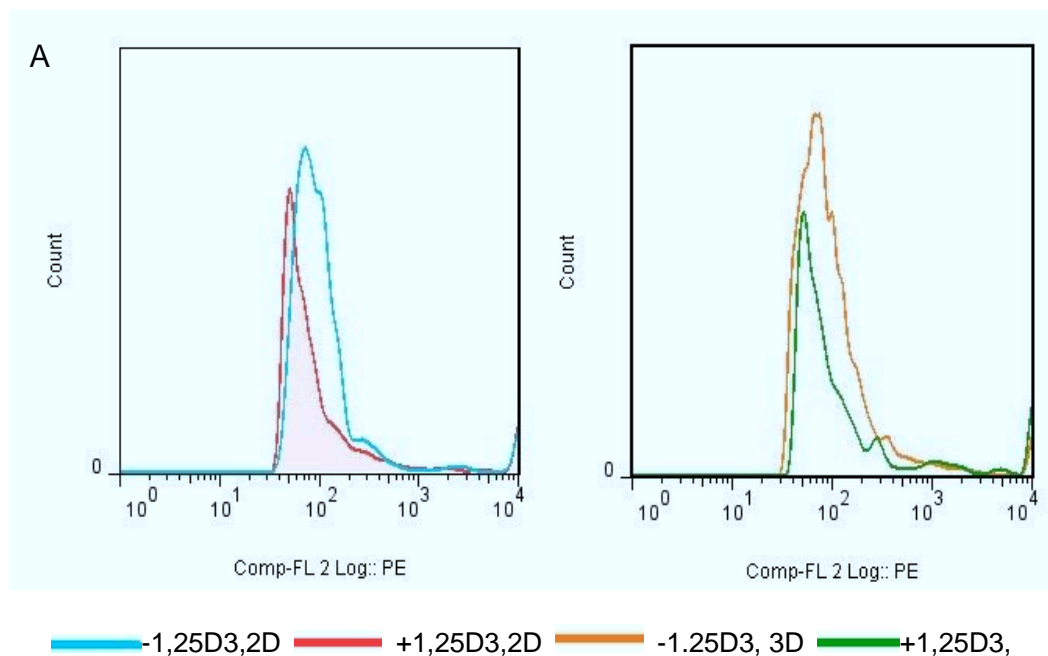
Figure 2. Migration Rate of vitamin D pre-treated and untreated breast cancer cells through a microporous membrane in the 2D and 3D system.

****p ≤ 0.0001**

2D: MDA-MB-231 cells in 2D osteoblast system; 3D: MDA-MB-231 cells in 3D osteoblast system; +1,25D3: 1, 25 dihydroxyvitamin D pre-treated breast cancer cells; -1,25D3: untreated breast cancer cells.

1, 25 dihydroxyvitamin D pre-treated breast cancer cells express less CXCR4 than untreated breast cancer cells

To identify a potential mechanism for the decreased metastatic ability of 1, 25 dihydroxyvitamin D pretreated breast cancer cells (2-4), the expression of CXCR4 was investigated since it is known to have a role in the migration and homing of several cancer cells including breast cancer (ref). The pre-treated and untreated MDA-MB-231 cells were cultured in 2D and 3D CM and then assess the cell surface expression of CXCR4 by flow cytometry. In both 2D and 3D CM, the expression of CXCR4 by vitamin D pre-treated breast cancer cells decreased significantly when compared with untreated breast cancer cells. The data are shown in Figure 3.



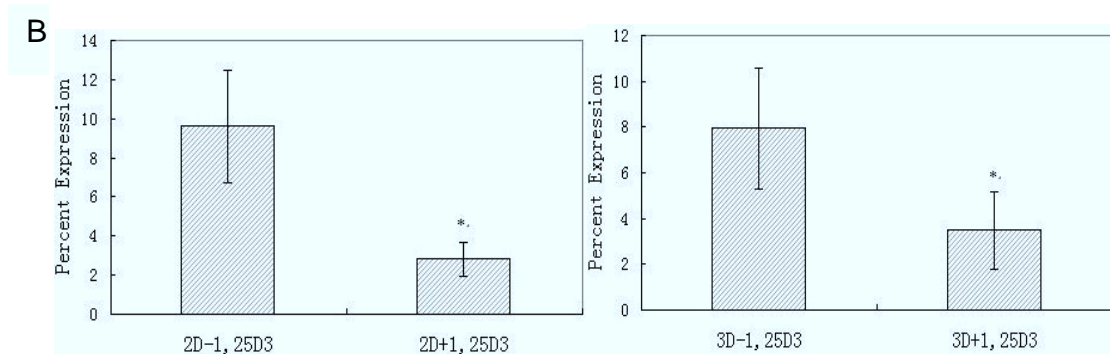
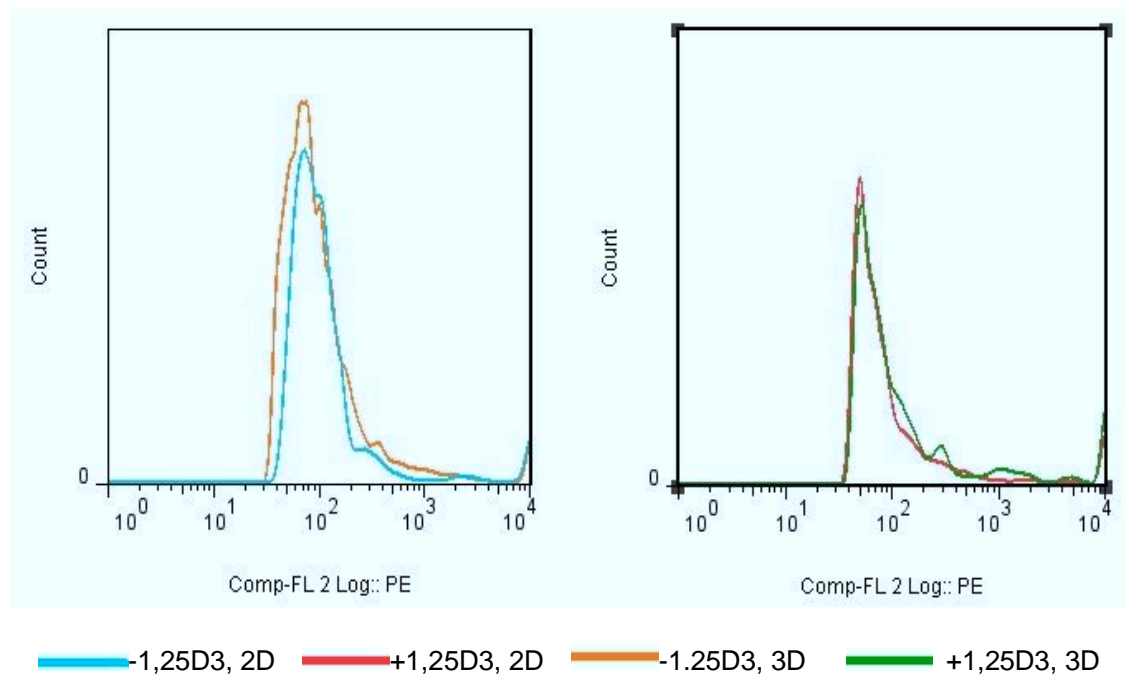


Figure 3. Comparison of vitamin D treated and untreated breast cancer cell surface expression of CXCR4. Human breast cancer cell line MDA-MB-231 were cultured complete medium with 100nM 1, 25 dihydroxyvitamin D₃, or complete medium for 48 hours. The pre-treated and untreated cells were exposed to 2D or 3D OBCM, respectively. Cell surface expression of CXCR4 was analyzed by FlowJo software (A). Data are expressed as the mean ± SD of the percent of cells express CXCR4. * $p \leq 0.05$ (B). 2D: MDA-MB-231 cells in conditioned medium from 2D osteoblast system; 3D: MDA-MB-231 cells in conditioned medium from 3D osteoblast system; +1,25D₃: 1, 25 dihydroxyvitamin D pre-treated MDA-MB-231 cells; -1,25D₃: untreated MDA-MB-231 cells.

Breast cancer cells do not express more CXCR4 when growing in 3D CM

The expression levels of CXCR4 in breast cancer cells growing in 2D and 3D CM were also compared. No significant differences in CXCR4 expression by either vitamin D pre-treated or untreated breast cancer cells were found. The data are shown in Figure 4.

A



B

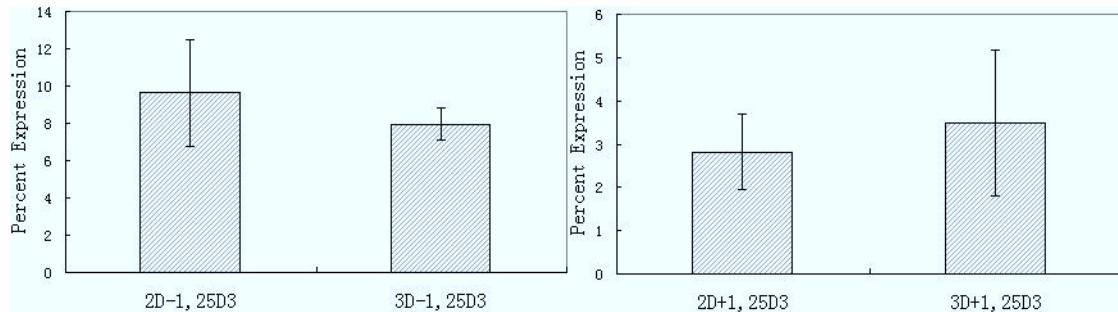


Figure 4. Comparison of breast cancer cell surface expression of CXCR4 in 2D and 3D conditioned medium. Human breast cancer cell line MDA-MB-231 were cultured complete medium with 100nM 1, 25 dihydroxyvitamin D₃, or complete medium for 48 hours. The pre-treated and untreated cells were exposed to 2D or 3D OBCM, respectively. Cell surface expression of CXCR4 was analyzed by FlowJo software (A). Data are expressed as the mean ± SD of the percent of cells express CXCR4. *p ≤ 0.05 (B)

**Treated BCCM (Breast cancer CM prepared from vitamin D treated cells)
does not decrease the expression of CXCL12 by osteoblast cells**

In order to ascertain whether CXCL12, the ligand of CXCR4 is increasingly expressed by osteoblast cells in response to the rising level of CXCR4, both 2D and 3D osteoblast cells were cultured in pre-treated and untreated BCCA CM for 2days, and qRT-PCR was used to detect changes in the expression of CXCL12 mRNA. We did not find any significant differences in CXCL12 expression by osteoblast cells in the presence of either pre-treated CM or untreated CM. The data are shown in Figure 5.

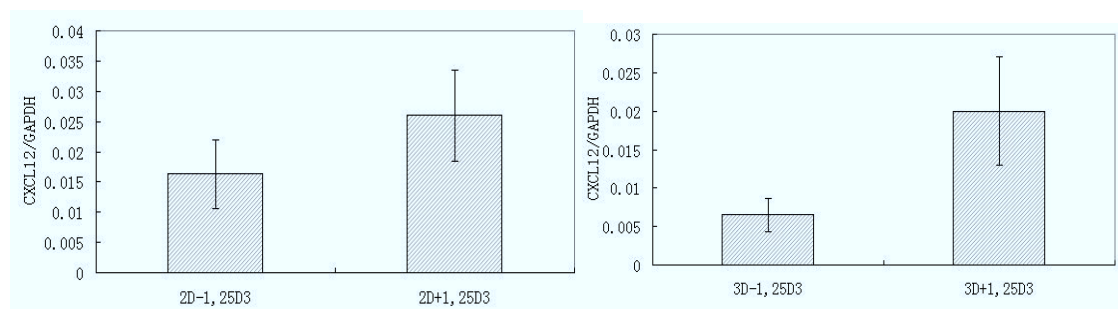


Figure 5. Gene expression in osteoblasts cultured in vitamin D pre-treated and untreated BCCM. 1, 25 dihydroxyvitamin D pretreated and untreated BCCM were used to cultured 2D osteoblast cells and 3D osteoblast cells, respectively. Transcript levels of CXCL12 in osteoblast cells were quantified by qRT-PCR and normalized to GAPDH. * $p \leq 0.05$

Osteoblasts growing in collagen scaffolds do not express more CXCL12

The expression of CXCL12 by 2D and 3D osteoblasts were also compared in this study. By quantitative real time PCR, we did not see any significant difference between 2D and 3D osteoblasts, either in pre-treated or untreated BCCM. The data are shown in Figure 6.

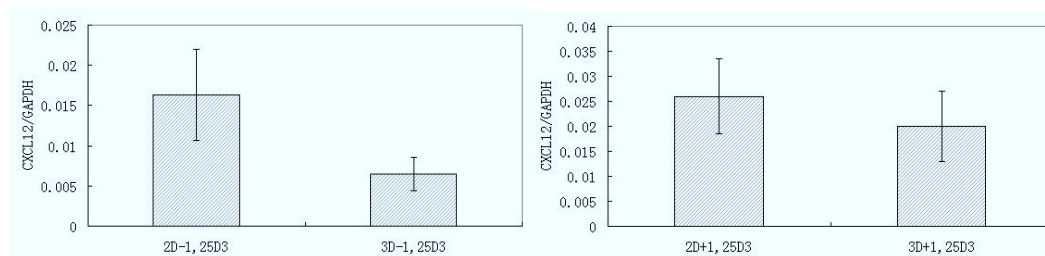


Figure 6. Gene expression in the MDA-MB-231 culturing in 2D and 3D microenvironment. 1, 25 dihydroxyvitamin D₃ pretreated and untreated BCCM were used to cultured 2D osteoblast cells and 3D osteoblast cells, respectively. Transcript levels of CXCL12 in osteoblast cells were quantified by qRT-PCR and normalized to GAPDH. *p ≤ 0.05

CHAPTER 5

DISCUSSION

To evaluate the breast cancer metastasis, we created 3D system by culturing the osteoblasts in collagen scaffolds, which provided a spatial culture microenvironment to mimic the in vivo situation, and compare the cancer migration in this system and in 2D cell-monolayer system. We found breast cancer cells migrated about twice in this 3D system than in the 2D microenvironment. It suggested that the 3D system is more sensitive for breast cancer migration and might be better for studying metastatic breast cancer.

There are existing studies revealing correlation between vitamin D and breast cancer metastasis (16, 19-22). In our study, we found in the 2D system, the rate of migrated 1, 25 dihydroxyvitamin D₃ pre-treated breast cancer cells was only 50% of the untreated cells. Similar results were also found in the 3D system as there was a 40% decrease rate. We conclude that 1, 25 dihydroxyvitamin D₃ could prevent breast cancer migration. We also compared the data from pre-treated breast cancer cells growing in 2D and 3D system. In the 3D microenvironment, vitamin D's anti-migration activity was weaker than that in 2D, for there were more migrated cells as well as the less decreasing migration rate. It seems likely that the collagen matrix in the microenvironment is also an important factor to influence the anti-tumor activity of 1, 25 dihydroxyvitamin D₃. It supports our hypothesis that the 3D system of osteoblasts growing in the supportive scaffolds may provide more protective and chemoattractive agents. These data

demonstrate that the vitamin D pre-treatment worked, and the 3D model worked better.

CXCR4 is important in breast cancer migration (25). In order to investigate whether the increased sensitivity of 3D system was caused by the increased expression of CXCR4 in breast cancer cells, and the inhibition of migration by 1, 25 dihydroxyvitamin D₃ was the result of suppressing expression of CXCR4, changes in the expression of CXCR4 of MDA-MB 231 cells cultured in the 2D and 3D osteoblast conditioned media were monitored. In our study, vitamin D pretreated breast cancer cells expressed less CXCR4 than untreated cells while having less migration rate. It indicates that vitamin D may suppress the migration of breast cancer by downregulating the cell surface expression of CXCR4 so it could be the direct target of vitamin D. No differences between the breast cancer cells growing in the 2D and 3D OBCM were seen. This might point out that CXCR4 is not a factor which is affected by the supporting matrix in bone microenvironment or there may be other cells or factors in the BME (which were not available in the current model) whose presence might be necessary to modulate the expression of CXCR4 in breast cancer cells.

As we hypothesized the increased migration rate was caused by the CXCR4-CXCL12 axis, we also detected the osteoblast expression of CXCL12 since it is the ligand of CXCR4 and theoretically the amount of it should be consistent with the expression of CXCR4. We observed that there were no significant differences in CXCL12 level between 2D and 3D osteoblasts culturing

in BCCM. It could possibly be the reason that the collagen supporting matrix could not increase the expression of CXCL12. Along with the non-significant difference between the breast cancer cells' expression of CXCR4 in the two systems, we can say the better sensitivity of 3D system is not the result of CXCR4-CXCL12 axis.

The breast cancer and osteoblast interact with each other not only by the CXCR4-CXCL12 axis, but some other factors as well. For example, the breast cancer cells can overproduce PTHrP, which can increase the osteolytic metastasis. Meanwhile, TGF- β , secreted from the bone matrix, stimulate the cancer cells to produce more PTHrP, thus establishing a positive feedback loop (26). PTHrP works in both RANKL dependent and independent mechanisms (27-30). In addition, IL-8 also belongs to the CXC chemokine family, is over expressed in MDA-MB-231 cell line (30). It can stimulate early metastasis by initiating bone resorption process (28). The higher sensitivity of the 3D system could possibly be due to these factors. Osteoblasts can produce some growth factors such as IGF, TGF- β , FGF, and BMPs, which may incorporated into bone matrix (2, 3). They are released into bone microenvironment and make it attractive to the cancer cells (31). As the scaffolds provide the collagen, one of the main component of bone extracellular matrix to the osteoblasts, there might be an accumulation of such factors in the matrix and when we co-culture the breast cancer cells with osteoblasts, osteolysis begins thus these factors are released and attract the cancer cells. It could be another potential explanation for the

sensitivity of 3D models.

Limitation

To our knowledge, this is the first model to create the 3D bone microenvironment for metastatic breast cancer with collagen matrix scaffolds. One limitation in the design could be that in this study, the 3D model only includes osteoblasts and extracellular matrix. However, bone microenvironment is complex and harbors other types of cells such as osteoclasts, marrow stromal cells, vascular endothelial cells, and immune cells. These cells interact with each other and mediate the signaling pathways that are critical for adhesion, migration, and homing of breast cancer cells in the bone microenvironment. The current 3D model does not completely justify the complexity of the *in vivo* bone microenvironment.

Another limitation of our study was the difficulty of quantifying the cells in the 3D system. Although the initial seeding densities in both models were the same, it is possible that with the support of collagen matrix, the osteoblasts grew better and proliferate faster in 3D tissue culture system. Along with the fact that it is difficult to measure the number of osteoblasts in each scaffold, we cannot exclude the fact that the difference in the migration rate was caused by the difference of osteoblast numbers in the lower chamber due to better proliferation rates in 3D vs. 2D system. For future studies, a proliferation assay such as MTS assay or Ki 67 immunohistochemical staining should be included to quantify proliferating osteoblasts in 2D vs. 3D systems. If there were much more osteoblasts in the

lower chamber in 3D system than the 2D one, the chemoattractive factors' concentration could be higher, which would increase the migration rate.

In addition, the method of collecting osteoblast conditioned media could be another limitation. We denied our hypothesis that the increased migration in the 3D model was the consequence of CXCR4-CXCL12 axis because we did not find the increased CXCR4 production in breast cancer cells culturing in 3D OBCM. However, our hypothesis could still be reasonable because according to a study performed by Junga et al, MC3T3-E1 secreted high levels of CXCL12 into media early in culture, and the levels decreased as matrix deposition and mineralization proceeded over time (32). Because the OBCM we used were collected at day 15, and at this time the osteoblasts were already highly differentiated and mineralized, the OBCM might not have enough CXCL12 so the breast cancer cells would not produce more CXCR4 in response to CXCL12.

Implications

In our current study, we established a novel 3D in vitro model of bone metastatic breast cancer, and found that it was much more sensitive than the traditional 2D tissue culture system. Although we did not find the potential mechanism which caused the higher sensitivity, the results suggest a practical strategy of using such model to support further studies of breast cancer bone metastasis. We also investigated vitamin D's ability to inhibit breast cancer migration by downregulating the production of CXCR4. It suggests that this model can also provides a relatively ideal approach to test new drugs during the process

of drug development and therapies related to this disease, as well as uncover the mechanisms. It also indicates that vitamin D is likely a preventative agent for breast cancer migration.

Future studies

In this study, we only find a phenomenon that the 3D model was more sensitive in causing breast cancer migration. Looking for an appropriate method to adjust the difference in osteoblast number between 2D and 3D models will be needed in future studies to exclude the confounding factors and strengthen our findings. The mechanisms behind the findings should also be included in the future studies to make it more convincing. As the OBCM could be a possible limitation, we can try to assess the effects of co-culture with breast cancer cells and osteoblast 2D vs. 3D instead of BCCM on CXCL12 expression.

As we have found that 100nM 1, 25 dihydroxyvitamin D3 could decrease the migration of breast cancer cells probably by downregulating the expression of CXCR4. However, we did not demonstrate that this downregulation was not the effects of the decreased cell viability caused by the vitamin D treatment. In the future study, assays testing breast cancer viability should be applied.

Conclusion

The lack of ideal *in vitro* tissue culture systems of breast cancer metastasis to bone is a significant problem in current breast cancer research. We compared a 3D tissue culture system with traditional 2D system for breast cancer metastasis, and proved that this 3D system was more sensitive. With this tissue culture

system, the impact of vitamin D on the migration of breast cancer cells was demonstrated, and the down regulation of CXCR4 expression was identified as a potential mechanism of inhibition by 1, 25 dihydroxyvitaminD₃.

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APPENDIX A
CRYSTAL VIOLET STAINING

Crystal Violet Staining

1. Prepare for staining by flattening the tips of 10-20 swabs by pressing the head of swab firmly against a clean surface. These will be used to remove the cells.
2. Using a clean disposable pipette tip for each well, remove medium from the chamber.
3. Transfer the chambers to a clean, empty well of the plate. Add 6 drops of solution to each well, incubate for 20 min.
4. Using the forceps, grasp and remove the chambers. Wash excess stain from the chamber by gently dipping several times into distilled water. Flick off excess water and allow to air dry.
5. Observe under the electronic microscope.